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(71) Applicant *(for all designated States except US)*: **NITTO DENKO CORPORATION** [JP/JP]; 1-1-2, Shimohozumi, Osaka, Ibaraki 567-8680 (JP).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **VAN, Sang** [US/US]; 3490 Atlas Street, San Diego, California 92111 (US). **ZHOU, Jiadong** [CN/US]; 10813 Terraza Floracion, San Diego, California 92127 (US). **FU, Xiaoli** [CN/US]; 1456 Portofino Drive, Vista, California 92081 (US). **YU, Lei** [CA/US]; 3682 Strata Drive, Carlsbad, California 92008 (US).

(74) Agent: **ALTMAN, Daniel, E.**; KNOBBE, MARTENS, OLSON & BEAR, LLP, 2040 Main Street, Fourteenth Floor, Irvine, California 92614 (US).

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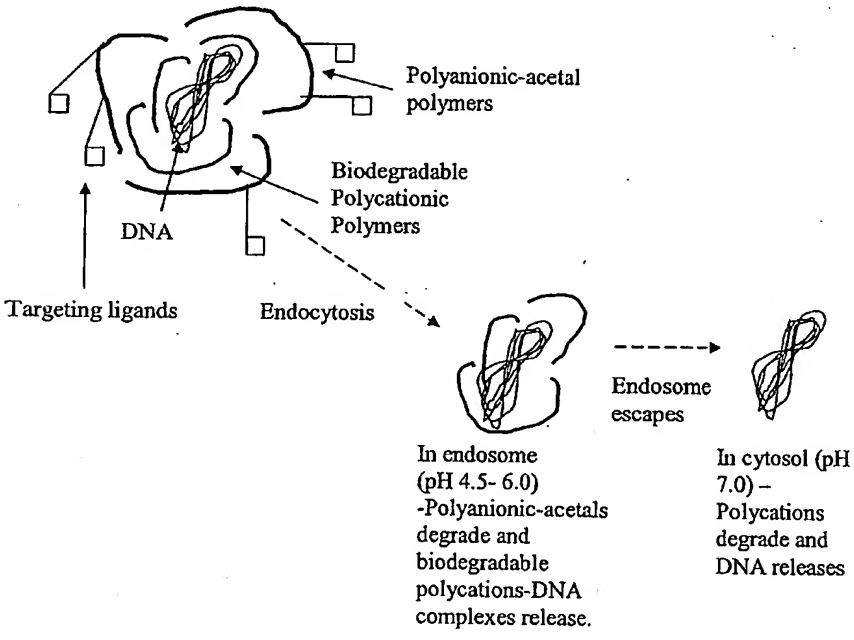

(57) Abstract: Degradable complexes comprising a polycation, a polyanion and a polynucleotide are useful for in vivo polynucleotide delivery applications.

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BIODEGRADABLE POLYACETALS FOR IN VIVO POLYNUCLEOTIDE DELIVERY

Related Application Information

This application claims priority to U.S. Provisional Patent Application No. 60/507,161, filed September 29, 2003, which is hereby incorporated by reference in its entirety.

Background of the Invention

Field of the Invention

This invention relates generally to degradable polymer compositions useful for *in vitro* and *in vivo* polynucleotide delivery applications. More particularly, this invention relates to degradable complexes comprising polyanions, polycations and polynucleotides useful for *in vivo* polynucleotide delivery applications in mammals.

Description of the Related Art

There is a need for non-viral drug delivery systems having desirable properties such as low immunogenicity, amenable to production on a relatively large scale, and which can be easily modified to provide a range of biological properties. See Mulligan, R. C., "The basic science of gene therapy," Science 260, 926-932 (1993); and Luo, D. & Saltzman, W. M. "Synthetic DNA delivery systems," Nat. Biotechnol. 18, 33-37 (2000). However, non-degradable cationic polymers such as poly(lysine) and polyethyleneimine (PEI) can have significant cytotoxicity. See Choksakulnimitr, S., Masuda, S., Tokuda, H., Takakura, Y. & Hashida, M., "In vitro cytotoxicity of macromolecules in different cell culture systems," J. Control Release 34, 233-241 (1995); Brazeau, G. A., Attia, S., Poxon, S. & Hughes, J. A., "In Vitro Myotoxicity of Selected cationic macrolecules used in non-viral gene therapy," Pharm. Res. 15, 680-684 (1998); and Ahn, C.-H., Chae, S. Y., Bae, Y. H. & Kim, S. W. "Biodegradable poly(ethylenimine) for plasmid DNA delivery," J. Control. Release 80, 273-282 (2002).

To reduce cytotoxicity, some efforts have been made to develop degradable cationic polymers (polycations). See Ahn, C.-H., Chae, S. Y., Bae, Y. H. & Kim, S. W., "Biodegradable poly(ethylenimine) for plasmid DNA delivery," J. Control. Release 80, 273-282 (2002); Lynn, D. M.; Anderson, D. G.; Putman, D.; Langer, R., "Accelerated

Discovery of Synthetic Transfection Vectors: Parallel Synthesis and Screening of a Degradable Polymer Library," J. Am. Chem. Soc. 123, 8155-8156 (2001); Lim, Y. et al., "Biodegradable Polyester, Poly[α-(4-Aminobutyl)-l-Glycolic Acid], as a Non-toxic Gene Carrier," Pharmaceutical Research 17, 811-816 (2000); Lim, Y., Kim, S., Suh, H. & Park, J.-S., "Biodegradable, Endosome Disruptive, and Cationic Network-type Polymer as a High Efficient and Non-toxic Gene Delivery Carrier," Bioconjugate Chem. 13, 952-957 (2002); Lim, Y. K., S.; Lee, Y.; Lee, W.; Yang, T.; Lee, M.; Suh, H.; Park, J., "Cationic Hyperbranched Poly(amino ester): A Novel Class of DNA Condensing Molecule with Cationic Surface, Biodegradable Three-Dimensional Structure, and Tertiary Amine Groups in the Interior," J. Am. Chem. Soc. 123, 2460-2461 (2001); and Tuominen, J. et al., "Biodegradation of Lactic Acid Based Polymers under Controlled Composting Conditions and Evaluation of the Ecotoxicological Impact," Biomacromolecules 3, 445-455 (2002). However, under physiological conditions, these cationic polymers are susceptible to degradation via base-catalyzed hydrolysis.

Acid-sensitive polymers containing acetal linkages have been reported, see Tomlinson, R. et al., "Pendent Chain Functionalized Polyacetals That Display pH-Dependent Degradation: A Platform for the Development of Novel Polymer Therapeutics," Macromolecules 35, 473-480 (2002); and Murthy, N., Thng, Y. X., Schuck, S., Xu, M. C. & Fréchet, J. M. J., "A Novel Strategy for Encapsulation and Release of Proteins: Hydrogels and Microgels with Acid-Labile Acetal Cross-Linkers," J. Am. Chem. Soc. 124, 12398-12399 (2002).

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U.S. Patent Application Serial No. 10/270,788, filed October 11, 2002 (published as U.S. Patent Publication No. 2003-0215395 A1), and U.S. Provisional Patent Application Serial No. 60/378,164, filed May 14, 2002.

U.S. Patent Application Serial No. 10/375,705, filed February 25, 2003 (published as U.S. Patent Publication No. 2004-0166089 A1).

Tomlinson R, Heller J, Brocchini S, Duncan R. "Polyacetal-doxorubicin conjugates designed for pH-dependent degradation." Bioconjug Chem. 2003, 14(6), 1096-1106.

Summary of the Invention

It is believed that binary polycation-DNA complexes enter cells by an endocytotic pathway as illustrated in Figure 6. However, it has been found that the ability of such binary complexes to deliver polynucleotides to cells *in vivo* is limited by toxicity and low gene expression. It is believed that decreased transfection efficiency *in vivo* is caused by interaction of the positively charged binary polycation-DNA complexes with negatively charged components such as proteins. The positive charges of the binary polycation-DNA complex can be shielded by treatment with polyethyleneglycol (PEG) or proteins such as transferrin, or tertiary complexes may be formed with polycations as shown in Figure 7.

However, in practice it has been found that such additional components tend to complicate the release and delivery of the DNA.

It has now been found that biodegradable tertiary complexes comprising a polyanion, a polycation and a polynucleotide, as shown in Figure 8, provide a number of benefits. Thus, a preferred embodiment provides a complex for delivering a polynucleotide to a cell, comprising a polynucleotide; a polycation; and an acid-degradable polyanion.

Another preferred embodiment provides a method of making such a complex, comprising intermixing a first solution comprising the acid-degradable polyanion with a second solution comprising the polycation and the polynucleotide.

Another preferred embodiment provides a method of delivering such a complex into a cell, comprising contacting the complex with the cell.

These and other embodiments are described in greater detail below.

Brief Description of the Drawings

These and other aspects of the invention will be readily apparent from the following description and from the appended drawings, which are meant to illustrate and not to limit the invention, and wherein:

Figure 1 shows a reproduction of a photograph showing the results of a retardation assay carried out using complexes formed from an acid degradable polyanion (polyacetal 5), a biodegradable polycation and DNA. The numbers above the photograph indicate the ratios of polycation to DNA and polyanion to DNA ratio (by weight/weight). The results show that, for this particular set of conditions, DNA was not released from the complex, as compared to a control C (no polymers) and a molecular marker M. The results show that acid degradable polyanion (polyacetal 5), biodegradable polycation and DNA formed a tertiary complex.

Figure 2 shows a bar graph plotting Relative Light Units (RLU) per milligram of protein for transfection of HepaG2 cells using a tertiary complex formed from an acid degradable polyanion (polyacetal 10), a biodegradable polycation, and DNA, as compared to a binary complex formed from the biodegradable polycation and DNA, without the anionic polymer. The results show that transfection using the tertiary complex was superior to transfection using the binary complex. The results indicate that the tertiary complex is likely to be effective for in vivo polynucleotide delivery. Labeling: Ratio of biodegradable

polycation:DNA (by weight/weight) for vertical line bar is 32:1, horizontal line bar is 16:1, and grid line bar is 8:1. The numbers on the horizontal axis are the ratio of polyacetal **10** to DNA (by weight/weight).

Figure 3 shows a preferred reaction scheme for the preparation of anionic polyacetals **4-9**.

Figure 4 shows a preferred reaction scheme for the preparation of anionic polyacetal **10**.

Figure 5 shows a preferred reaction scheme for the preparation of biodegradable polycation **12**.

Figure 6 schematically illustrates an endocytoxic pathway for the entry of a binary polycation-DNA complex into a cell.

Figure 7 schematically illustrates a tertiary complex of polyanion, polycation and polynucleotide.

Figure 8 schematically illustrates a biodegradable tertiary complex of polyanion, polycation and polynucleotide.

Figure 9 shows a preferred reaction scheme for the preparation of anionic polyacetal **13** as described in Example 11.

Detailed Description of the Preferred Embodiments

A preferred embodiment is directed to a tertiary complex comprising a polyanion, a polycation and a polynucleotide, wherein at least one of the polyanion and the polycation is biodegradable (e.g., acid-degradable). Preferably, the polycation is biodegradable and the polyanion is acid-degradable as illustrated in Figure 8. Preferably, the polynucleotide is DNA or RNA. Non-limiting examples of preferred polynucleotides include plasmid DNA, antisense DNA, DNA oligomer, siRNA, ribozyme, and tetramer.

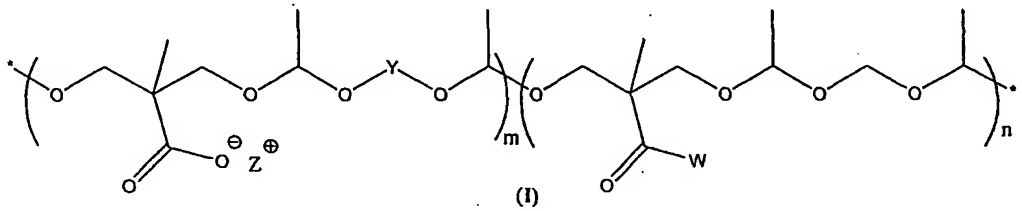
Polycations are macromolecules with multiple groups that are positively charged or capable of being positively charged under physiological or acidic conditions. A quaternary amine is an example of a cationic group; primary, secondary and tertiary amines are examples of groups that are capable of being positively charged under physiological or acidic conditions. Examples of polycations include polyamine and poly(ethylenimine). The polycation is typically associated with a counterion such as a negatively charged group on a polyanion, an organic ion, or an inorganic ion such as fluoride, chloride, bromide,

iodide, nitrate, or sulfate. Preferred biodegradable polycations comprise one or more degradable recurring units that undergo degradation under physiological conditions, preferably resulting in scission of the polycation backbone to form lower molecular weight fragments. Non-limiting examples of preferred degradable recurring units include ester, amide, phosphatediester, acetal, imine, hydrazone, enol, enol ether, enamine, ketal, and oxime. Non-limiting examples of preferred biodegradable polycations thus include polyester-polyamine, polyphosphatediester-polyamine, and polyacetal-polyamine. Biodegradable polycations may be purchased from commercial sources or prepared by methods known to those skilled in the art. Preferred polycations are preferably prepared as described in U.S. Patent Application Serial No. 10/270,788, filed October 11, 2002 (published as U.S. Patent Publication No. 2003-0215395 A1), and U.S. Provisional Patent Application Serial No. 60/378,164, filed May 14, 2002, both of which are hereby incorporated by reference in their entireties and particularly for the purpose of describing the preparation of degradable polycations. The molecular weight of polycations is preferably in the range of about 500 daltons to about 5,000,000 daltons, more preferably in the range of about 2,000 daltons to about 50,000 daltons.

Polyanions are macromolecules with multiple groups that are negatively charged. Carboxylate (CO_2^-), sulfonate (SO_3^-), and phosphate (PO_3^-) are non-limiting examples of groups that are negatively charged. The polyanion is typically associated with a counterion such as a cationic group on a polycation, an ammonium ion (NH_4^+), or a metal ion such as Li^+, Na^+, K^+, Rb^+, Cs^+, Be^{2+}, Mg^{2+}, or Ca^{2+}. Preferred biodegradable polyanions comprise one or more degradable recurring units that undergo degradation under physiological conditions, preferably resulting in scission of the polycation backbone to form lower molecular weight fragments. Preferred biodegradable polyanions are acid-degradable. Non-limiting examples of preferred degradable recurring units include ester, amide, phosphatediester, acetal, imine, hydrazone, enol, enol ether, enamine, ketal, and oxime. Biodegradable polyanions may be purchased from commercial sources or prepared by methods known to those skilled in the art. Preferred polyanions are polyacetals prepared by hydrolysis of the corresponding polyacetal esters as illustrated in Figure 3. The polyacetal esters are preferably prepared as described in U.S. Patent Application Serial No. 10/375,705, filed February 25, 2003 (published as U.S. Patent Publication No. 2004-0166089 A1), which is hereby incorporated by reference in its entirety and particularly for

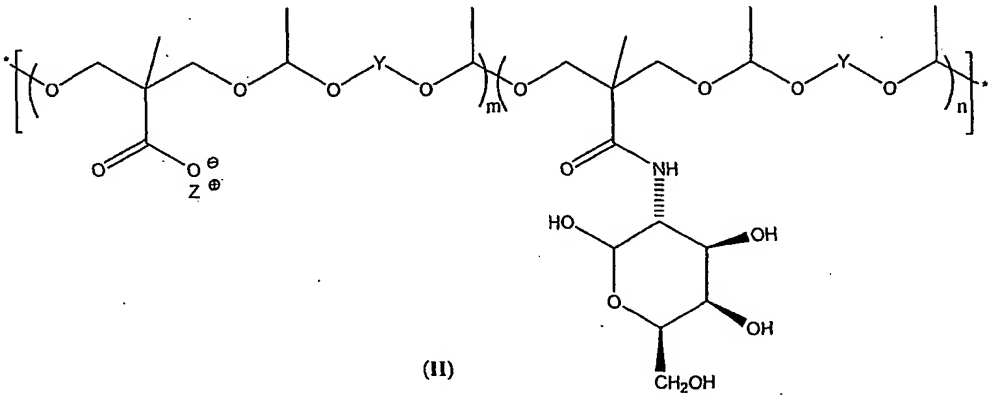
the purpose of describing the preparation of degradable polyacetals. The molecular weight of polyanions is preferably in the range of about 500 to about 5,000,000.

In particularly preferred embodiments, biodegradable polyanions comprise a targeting ligand, preferably a targeting ligand selected from the group consisting of galactose, lactose, mannose, peptide, antibody, antibody fragment, and transferrin. Particularly preferred polyanions comprise recurring units represented by Formula (I):



(I)

wherein Y is selected from the group consisting of $-(CH_2)_2-$, $-(CH_2)_2-O-(CH_2)_2-$, and $-(CH_2)_2-O-(CH_2)_2-O-(CH_2)_2-$; wherein W is a targeting ligand selected from the group consisting of galactose, lactose, mannose, transferrin, antibody, antibody fragment, and RGD peptide; wherein m and n are each individually integers in the range of 1 to 100,000; and wherein Z is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, and Ca. For example, a highly preferred polyanion comprises recurring units represented by formula (II)



(II)

wherein Y is selected from the group consisting of $-(CH_2)_2-$, $-(CH_2)_2-O-(CH_2)_2-$, and $-(CH_2)_2-O-(CH_2)_2-O-(CH_2)_2-$; wherein m and n are each individually integers in the range of one to 100,000; and wherein Z is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, and Ca. A polyanion comprising recurring units represented by formula (II) is

preferably prepared by hydrolyzing the corresponding polyacetal precursor to form a hydrolyzed polyacetal; and coupling a galactosamine with the hydrolyzed polyacetal to form the polyanion. The coupling is preferably conducted using a coupling reagent selected from the group consisting of 1,3-diisopropylcarbodiimide (DIC), N-hydroxysuccinimide (NHS), and 4-dimethylaminopyridine (DMAP). The polyacetal precursor is preferably prepared as described in U.S. Patent Application Serial No. 10/375,705, filed February 25, 2003, which has been incorporated by reference herein as discussed above.

Biodegradable tertiary complexes comprising a polyanion, a polycation and a polynucleotide may be prepared in various ways. Preferably, the polyanion, polycation and polynucleotide are dissolved in solution and intermixed to form a tertiary complex. More preferably, a the polycation and the polynucleotide are intermixed in solution, preferably forming a positively charged binary complex, and the resulting solution is then preferably intermixed with the polyanion to form a tertiary complex. The ratios of polycation to polynucleotide and polyanion to polynucleotide may vary over a broad range. Preferably, the ratio of polycation to polynucleotide is in the range of about 1:1 to about 100:1, more preferably in the range of about 5:1 to 50:1.

The ratio of polyanion to polynucleotide is preferably selected by taking into consideration the polycation to polynucleotide ratio, and utilizing an amount of polyanion that is effective to at least partially neutralize any excess of cationicity present in a binary complex formed between the polycation and polynucleotide. For example, the amount of polycation is preferably in excess of the amount of polynucleotide on a weight basis, and thus the amount of cationic charge on the polycation is often in excess of the amount of negative charge on the polynucleotide. Thus, any binary complex formed by using such amounts of polycation and polynucleotide will typically have a net positive charge. Preferably, the amount of polyanion used with such amounts of polycation and polynucleotide is effective to at least partially neutralize the net positive charge. More preferably, the polyanion is used in an amount that slightly exceeds the amount effective to neutralize the net positive charge. Those skilled in the art recognize that the relative amounts of polycation, polyanion and polynucleotide needed to obtain a tertiary complex having the desired nucleotide content and charge level may be calculated in advance based on the known or determined charge levels of the components, and thus formation of a

binary polycation-polynucleotide complex is not required prior to the addition of the polyanion.

In vitro and *in vivo* delivery of the polynucleotide to the interior of a cell (transfection) may be carried out by contacting the tertiary complex with the cell to be transfected. Preferably, the cell is a mammalian cell. The contacting of the tertiary complex with the cell *in vitro* may be carried out in various ways known to those skilled in the art, e.g., as described in the non-limiting examples set forth below. Contacting of the tertiary complex with the cell *in vivo* is preferably carried out by introducing the complex into the body of an animal, preferably a mammal, e.g., by systemic or local administration. Preferably, administration is conducted by identifying a mammal, e.g., a human, having cells in need of transfection with a particular polynucleotide, then administering a tertiary complex comprising the polynucleotide to the mammal in an amount effective to deliver the desired amount of polynucleotide to the cells. Such amounts of the tertiary complex effective to deliver desired polynucleotide may be determined by routine in vitro experimentation and/or by other methods known to those skilled in the art, e.g., clinical trials.

EXAMPLES

Cell lines and cultures used in the following examples were prepared as follows: Hepatoma (HepaG2) cells were grown in Dulbecco's-modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml Penicillin and 100 μg/ml streptomycin, and incubated at 37°C at 100% humidity atmosphere containing 7.5% CO_2. The plasmid vector pCMV-luc was constructed by cloning the firefly luciferase gene into pCMV-0, with the same backbone of mammalian expression vector. The plasmid was expanded in DH5α *E. coli* and purified with Qiagen Plasmid Max Preparation Kit according to the manufacture's instructions. The quantity and quality of the purified plasmid DNA was assessed by spectrophotometric analysis at 260 and 280 nm as well as by electrophoresis in 0.8% agarose gel. Purified plasmid DNA was resuspended in sterile distilled, deionized H_2O and frozen. Polyacetals **1-3** and biodegradable polycation **12** were synthesized in the manner described in U.S. Patent Application Serial No. 10/270,788, filed October 11, 2002 (published as U.S. Patent Publication No. 2003-0215395 A1); U.S. Provisional Patent Application Serial No. 60/378,164, filed May 14, 2002; and U.S. Patent

Application Serial No. 10/375,705, filed February 25, 2003 (published as U.S. Patent Publication No. 2004-0166089 A1). All of the chemicals and reagents for syntheses of polyacetals and biodegradable polycations were purchased from commercial sources. Poly(ethylenimine)-600 daltons (PEI-600) was purchased from Polysciences, Inc. Polymer molecular weights were measured by aqueous high pressure size exclusion chromatography (HPSEC) using polyethylene glycol standards. Sizes and zeta potentials of binary and tertiary complexes were analyzed by ZetaPALS (zeta potential and particle size analyzer) by following the protocols provided by the manufacturer (Brookhaven Instruments Corporation) Buffers and PEI (branched, 25k Daltons, Sigma-Aldrich) were obtained commercially.

EXAMPLES 1-3

Synthesis of polyacetals **4-6** (Figure 3): The following description for the synthesis of anionic polyacetal **5** is illustrative: A solution of lithium hydroxide monohydrate (0.45 g, 10.7 mmol) in methanol/water (1:1) (20 mL) was added to polyacetal **2** (2.57 g, 8.6 mmol). The reaction mixture was stirred for 24 hours and concentrated by rotary evaporation. The residue was redissolved in ethanol (50 mL). Acetone (200 mL) was added to the resulting solution to form a precipitate. The mixture was decanted and the residue was placed under high vacuum to give polyanion **5** (1.7 g).

EXAMPLES 4-6

Synthesis of polyacetals **7-9** (Figure 3): The following description for the synthesis of polyacetal **8** is illustrative: A solution of 1,3-diisopropylcarbodiimide (DIC, 0.082 g, 0.65 mmol) in DMF (10 mL) was added to anionic polyacetal **5** (0.39 g, 1.3 mmol). N-hydroxysuccinimide (NHS, 0.075 g, 0.65 mmol) in DMF (15 mL) was added into the reaction mixture. The reaction was stirred for 7 hours. The precipitate was isolated and washed with more DMF and placed under high vacuum to give **8** (0.45 g).

EXAMPLE 7

Synthesis of polyacetal **10**: A solution of galactosamine-HCl (0.11 g, 0.51 mmol) and 4-dimethylaminopyridine (DMAP, 0.12 g, 0.98 mmol) in dimethylsulfoxide (DMSO, 2 mL) was added to a solution of polyanion **8** (0.20 g) in PBS (8 mL). The reaction mixture

was stirred for 15 hours. The reaction solution was added with acetone (200 mL) and stirred. Within 1 hour, precipitate formed. The precipitate was redissolved in distilled water (2 mL) and dialyzed in distilled water (2000 mL) for 5 hours. The solution was placed under high vacuum to give **10**.

EXAMPLE 8

The polycation **12** used in the following examples was prepared as described in U.S. Patent Application No. 10/270,788, filed October 11, 2002 (published as U.S. Patent Publication No. 2003-0215395 A1), and U.S. Provisional Patent Application No. 60/378,164, filed May 14, 2002, as illustrated in Figure 5.

EXAMPLE 9

Tertiary complexation of polyacetal **5** to form DNA/polycation complexes: Samples of plasmid DNA and biodegradable polycation **12** were diluted in Optimem solution (Life Technologies) and mixed to form a series of binary polycation/DNA complexes having polycation to DNA ratios ranging from 32:1 to 8:1 by weight. After about 15 minutes, a solution of polyanion **5** in Optimem solution was added into the polycation/DNA complexes and incubated at room temperature for another 15 minutes. The resulting tertiary complexes were added to agarose gel and electrophoresis was performed: Five μl of loading dye was added to each sample, and 15 μl of each sample were loaded per well. The tertiary complexes were analyzed by electrophoresis in a 0.3% agarose gel with 0.04 M Tris-acetate buffer, pH 7.4, containing 1 mM EDTA, at 100V for 30 minutes. The tertiary complexes were visualized by UV illumination. The retardation assay results for the tertiary polycation/polyanion/DNA complexes are shown in Figure 1. The results indicate that the tertiary complexes were successfully formed and that polyanion **5** did not compete with DNA, so that DNA was released from the tertiary complexes at each of the ratios.

EXAMPLE 10

In vitro transfection using a tertiary complex comprising polycation **12** and anionic polyacetal **10** was carried out as follows: HepaG2 cells were plated in 24-well tissue culture plates ($2x10^5$ cells/well for HepaG2 cells) and incubated overnight in DMEM with 10% fetal bovine serum (FBS). For each well, a 20 μl aliquot of polycation **12** solution

(each containing a different dose of biodegradable polycation **12**) was added dropwise into a 20-μl DNA solution containing 0.6 μg of plasmid DNA (pCMV-GFP plasmid DNA or pCMV-luc), while vortexing. Dropwise addition while vortexing was found to be highly preferable, because it was found that transfection results depended on the mixing conditions. The solutions containing DNA and polycation **12** were incubated for 15 min at room temperature to form binary DNA-polycation **12** complexes. Then 20 ml of solutions containing anionic polyacetal **10** were added to each of the binary DNA-polycation **12** complexes to form tertiary complexes (containing DNA, anionic polyacetal **10**, and polycation **12**). Next, 60 mL samples of these tertiary complexes were added into each well and the cells were incubated (37° C, 7.5% CO_2) for 24 hours. After that incubation, fruitfly luciferase activities were detected as described below.

Luciferase activity was measured using a chemiluminescent assay following the manufacturer's instructions (Luciferase Assay System, Promega, Madison, Wisconsin, USA). About twenty four hours after the transfections described above, the cells were rinsed twice with PBS and then were lysed with lysis buffer (1% Triton X-100, 100 mM K_3PO_4, 2 mM dithiothreitol, 10% glycerol, and 2 mM EDTA pH 7.8) for 15 min at room temperature. A 10-μl aliquot of cell lysate was then mixed with 50-μl of luciferase assay reagent with the injector at room temperature in the luminometer. Light emission was measured in triplicate over 10 seconds and expressed as RLUs (relative light units). Relative light units (RLU) were normalized to the protein content of each sample, determined by BSA protein assay (Pierce, Rockford, Illinois). All the experiments were conducted in triplicate. The results obtained for the transfection of HepaG2 cells with pCMV-luc using the tertiary complexes (containing DNA, anionic polyacetal **10**, and polycation **12**) are shown in Figure 2. The results show that tertiary complexes containing polyacetal **10** provided increased transfection efficiency. The results also indicated that the tertiary complexes would be effective for *in vivo* polynucleotide delivery.

EXAMPLE 11

Synthesis of polyacetal **13** (Figure 9): 1,2,4,5-Benzenetetracarboxylic dianhydride (5.0 g, 22.9 mmol, Aldrich Chemical Company) was added to a solution of 3,9-bis(3-aminopropyl)-2,4,8,10-tetraoxaspiro[5.5]undecane (6.34 g, 22.9 mmol TCI Chemical Company) in dimethylacetamide (DMA, 100 mL,). The mixture was stirred for 3 days at

room temperature. The reaction was quenched with saturated sodium bicarbonate carbonate in water (50 mL). The precipitate was filtered, and the filtrate was poured in acetone (1000 mL) and white precipitate formed. The precipitate was redissolved in distilled water and dialyzed in cellulose membrane (2,000 molecular weight cut-off) in distilled water for 15 hours with 4 times of water changing. Clear solid polyacetal **13** (3.2 g) was obtained after the water was removed by rotary evaporation. The molecular weight of the polyacetal **13** was about 45,000 daltons (by HPSEC, polyethylene glycol standards). ^{1}H- and ^{13}C NMR spectra of polyacetal **13** were obtained and found to be consistent with the chemical structure of polyacetal **13** shown in Figure 9. In Figure 9, n and m are each individually integers in the range of about 1 to about 200. Control over the molecular weight of the polyacetal **13** (and thus over m and n) may be exercised by varying the molar ratio of the dianhydride monomer to the diamine monomer.

EXAMPLE 12

Sizes and zeta potentials of binary polycation PEI-DNA and biodegradable polycation **12**-DNA complexes were determined as follows:

Size Measurements: A series of solutions containing different amounts of polycation PEI or biodegradable polycation **12** (50 μL) in HEPES buffers was added to solutions containing DNA (2 μg, 50 μL) in HEPES buffers by pipetting, agitated by pipetting, then allowed to equilibrate for 1 minute. Particle sizes (nm) of the resulting binary complexes were measured by ZetaPALS, and the results are summarized in Table 1 below.

Zeta potential measurements: Selected different amounts of PEI or polycation **12** were added to a solution of DNA with similar concentrations as in the size measurements but scaled-up to 1.6 mL total volume. Zeta potentials (mV) of the resulting binary complexes were measured by ZetaPALS, and the results are summarized in Table 1 below.

Table 1

	Amount of PEI (μg)												
	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512
PEI:DNA ratio	0.06	0.1	0.25	0.5	1	2	4	8	16	32	64	128	256

(w:w)													
Particle size (nm)	124.1	103	153	112	94.6	78.6	82.5	88.1	80.4	69	55.8	63.6	91
Zeta potential (mV)			-29		49						51		
Poly-dispersity	0.311	0.21	0.11	0.2	0.21	0.23	0.21	0.27	0.25	0.2	0.33	0.3	0.3
Avg. Count Rate (kcps)	23.9	73.4	497	335	203	143	158	167	161	151	169	200	305
Baseline Index	0	2.7	7.8	6.5	10	9.7	8.6	0	0	9.8	8.2	7.2	9.9
	Amount of biodegradable polycation 12 (μg)												
		0.25	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	
12:DNA ratio (w:w)		0.1	0.25	0.5	1	2	4	8	16	32	64	128	
Particle size (nm)		200	106	110	1316	91.2	88.7	80.4	77.3	74	72.8	71.3	
Zeta potential (mV)				-27	19	38.2							
Poly-dispersity		0.3	0.21	0.1	0.3	0.12	0.13	0.19	0.19	0.2	0.2	0.2	
Avg. Count Rate (kcps)		18.1	61.9	227	277	274	259	162	143	126	121	118	
Baseline Index		0	5.1	8.3	9.1	8.9	8.9	7.8	9.3	9.4	9.8	8.3	

EXAMPLE 13

Sizes and zeta potentials of tertiary complexes were determined as follows:

Size Measurements: A series of solutions containing different amounts of polyacetal 13 (100 μL) in buffers were added to solutions containing binary PEI and DNA complexes (8 μg and 2 μg, respectively, 100 mL) in HEPES buffers by pipetting, agitated by pipetting, then allowed to equilibrate for 1 minute. Particle sizes (nm) of the resulting

ternary complexes were measured by ZetaPals, and the results are summarized in Table 2 below.

Zeta potential measurements: A series of solutions containing different amounts of polyacetal 13 in buffers were added to solutions of binary PEI and DNA complexes with similar concentrations as in the size measurements but scaled-up to 1.6 mL total volume. Zeta potentials (mV) of the resulting ternary complexes were measured by ZetaPals, and the results are summarized in Table 2 below.

Table 2

	Amount of Polyacetal 13 (μg) (HEPES buffer)					
	500	250	125	64	32	16
13:PEI:DNA (w/w)	500:8:2	250:8:2	125:8:2	64:8:2	32:8:2	16:8:2
measurement 1 (nm)	177	146	138	140	Turbidity	121
measurement 2 (nm)	182	145	137	143	Turbidity	99
measurement 3 (nm)	173	154	136	143	Turbidity	114
zeta (mV)	-37	-51	-28	-25		42
	Amount of Polyacetal 13 (μg) (BIGS buffer)[1]					
	500	250	125	64	32	16
13:PEI:DNA (w/w)	500:8:2	250:8:2	125:8:2	64:8:2	32:8:2	16:8:2
measurement 1 (nm)	183.6	170.1	148.1	150.6	Turbidity	157.8
measurement 2 (nm)	185.2	153.0	157.5	143.0	Turbidity	131.2
measurement 3 (nm)	178.4	161.1	154.0	166.5	Turbidity	142.2
zeta (mV)	-34	-30	-25	-32	-12	38

[1] BIGS buffers are 10% HEPES, 5% glucose, pH 7.4

EXAMPLE 14

Safety studies of the polyacetal 13: 15 hairless mice (SKH1 model, 5-6 weeks old, 18-20 grams) were purchased from Charles River Laboratories and were kept at the animal facility for 3 days before conducting experiments. 15 mice were divided into 3 groups (5 mice per group). Polyacetal 13 samples were prepared by dissolving the polymer in PBS (pH 7.4) at three different concentrations: (1) 40 mg/mL, (2) 20 mg/mL, and (3) 10 mg/mL). The mice were then injected (tail vein) with 100 μL of each concentration (one injection per mouse). The mice were observed for 5 hours and results are shown in Table 3 below:

Table 3

	Polyacetal 13 Dose (μg)		
	1,000	**2,000**	**4,000**
# of animals injected	5	5	5
# of animals alive	5	5	5
Behavior	Active	Active	Active

It will be appreciated by those skilled in the art that various omissions, additions and modifications may be made to the processes and compositions described above without departing from the scope of the invention, and all such modifications and changes are intended to fall within the scope of the invention, as defined by the appended claims.

WHAT IS CLAIMED IS:

1. A complex for delivering a polynucleotide to a cell, comprising:

a polynucleotide;

a polycation; and

an acid-degradable polyanion.

2. The complex of Claim 1 in which the polynucleotide is selected from the group of consisting of DNA and RNA.

3. The complex of Claim 1 in which the polynucleotide is selected from the group of consisting of plasmid DNA, antisense DNA, DNA oligomer, siRNA, ribozyme, and tetramer.

4. The complex of any preceding claim in which the polycation has a weight average molecular weight in the range of about 2,000 daltons to about 50,000 daltons as determined by high pressure size exclusion chromatography.

5. The complex of any preceding claim in which the polycation is selected from the group consisting of polyamine, poly(ethylenimine), polyester-polyamine, polyphosphatediester-polyamine, and polyacetal-polyamine.

6. The complex of any preceding claim in which the polycation is a biodegradable polycation.

7. The complex of Claim 6 in which the biodegradable polycation is selected from the group consisting of polyester-polyamine, polyphosphatediester-polyamine, and polyacetal-polyamine.

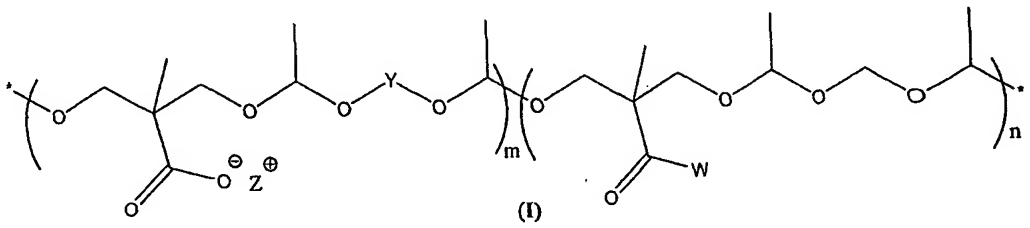
8. The complex of any preceding claim in which the acid-degradable polyanion comprises an acid-degradable group selected from the group consisting of acetal, imine, hydrazone, enol, enol ether, enamine, ketal, and oxime.

9. The complex of any preceding claim in which the acid-degradable polyanion comprises an anionic group selected from the group of consisting of carboxylate, sulfonate, and phosphate.

10. The complex of any preceding claim in which the acid-degradable polyanion comprises a targeting ligand.

11. The complex of Claim 10 in which the targeting ligand is selected from the group of consisting of galactose, lactose, mannose, peptide, antibody, antibody fragment, and transferrin.

12. The complex of any preceding claim in which the acid-degradable polyanion comprises recurring units represented by Formula (I):



(I)

wherein Y is selected from the group consisting of $-(CH_2)_2-$, $-(CH_2)_2-O-(CH_2)_2-$, and $-(CH_2)_2-O-(CH_2)_2-O-(CH_2)_2-$;

wherein W is a targeting ligand selected from the group consisting of galactose, lactose, mannose, transferrin, antibody, antibody fragment, and RGD peptide;

wherein m and n are each individually integers in the range of 1 to 100,000; and

wherein Z is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, and Ca.

13. A method of making the complex of any preceding claim, comprising:

intermixing a first solution comprising the acid-degradable polyanion with a second solution comprising the polycation and the polynucleotide.

14. A method of delivering a complex into a cell comprising contacting the complex of any of Claims 1-12 with a cell.

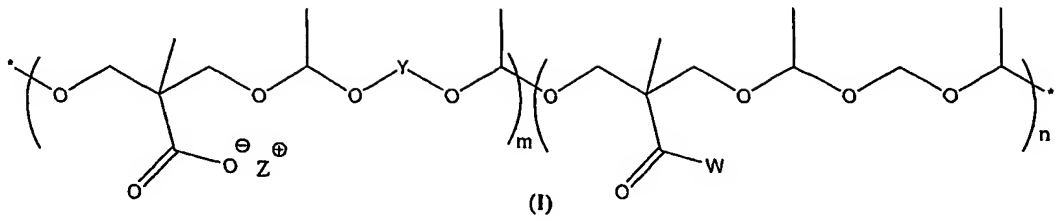
15. The method of Claim 14 in which the cell is a mammalian cell.

16. The method of Claim 14 or 15 in which the contacting of the complex with the cell is conducted by introducing the complex into the body of a mammal.

17. The method of Claims 16 in which the introducing of the complex into the body of the mammal is conducted by systemic administration.

18. The method of Claim 16 in which the introducing of the complex into the body of the mammal is conducted by local administration.

19. A polyanion comprising recurring units represented by Formula (I):



(I)

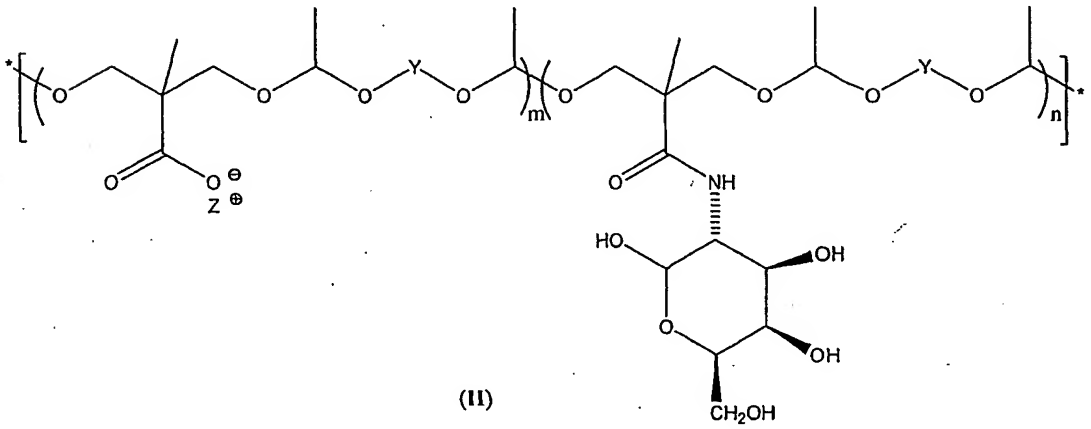
wherein Y is selected from the group consisting of $-(CH_2)_2-$, $-(CH_2)_2-O-(CH_2)_2-$, and $-(CH_2)_2-O-(CH_2)_2-O-(CH_2)_2-$;

wherein W is a targeting ligand selected from the group consisting of galactose, lactose, mannose, transferrin, antibody, antibody fragment, and RGD peptide;

wherein m and n are each individually integers in the range of one to 100,000; and

wherein Z is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, and Ca.

20. A polyanion comprising recurring units represented by formula (II):



(II)

wherein Y is selected from the group consisting of $-(CH_2)_2-$, $-(CH_2)_2-O-(CH_2)_2-$, and $-(CH_2)_2-O-(CH_2)_2-O-(CH_2)_2-$;

wherein m and n are each individually integers in the range of one to 100,000; and

wherein Z is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, and Ca.

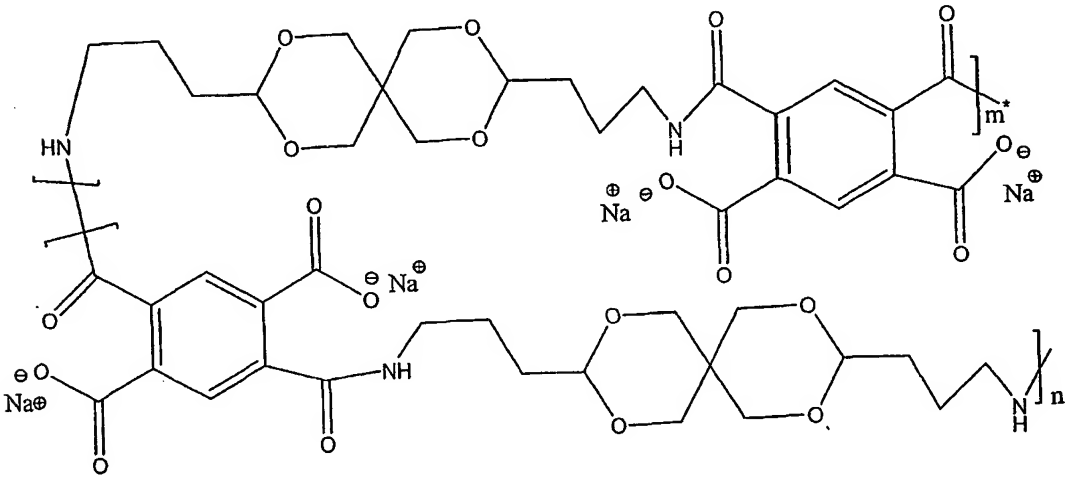
21. A method for making the polyanion of Claim 20, comprising:

hydrolyzing a polyacetal to form a hydrolyzed polyacetal; and

coupling a galactosamine with the hydrolyzed polyacetal to form the polyanion of Claim 20.

22. The method of Claim 21 in which the coupling is conducted using a coupling reagent selected from the group consisting of DIC, NHS, and DMAP.

23. A polyanion comprising recurring units represented by Formula (III):



(III)

wherein n and m are each individually integers in the range of about 1 to about 200.

FIGURE 1

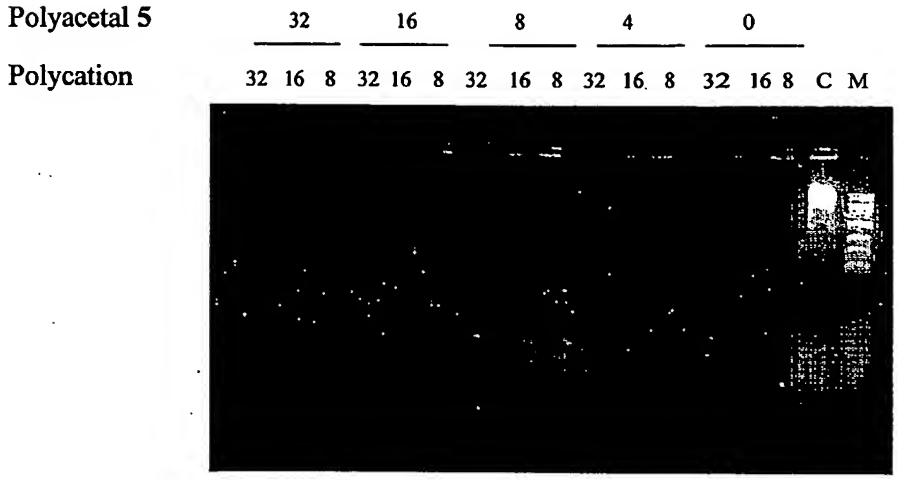

Polyacetal 5
32
16
8
4
0
Polycation
32 16 8 32 16 8 32 16 8 32 16 8 32 16 8 C M

FIGURE 2

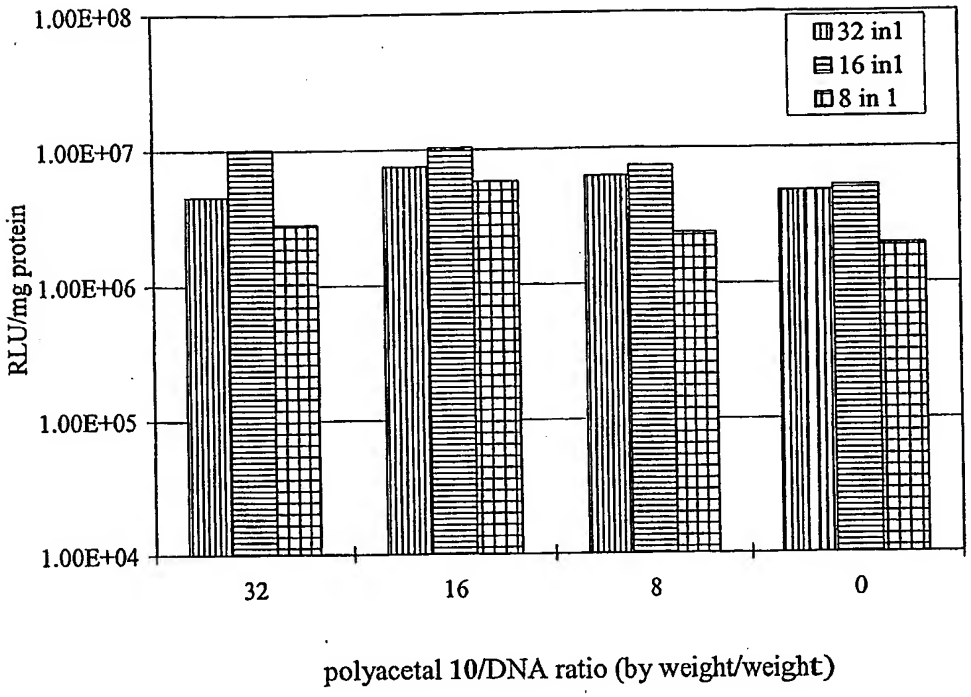

1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
RLU/mg protein
32 in1
16 in1
8 in 1
32
16
8
0
polyacetal 10/DNA ratio (by weight/weight)

FIGURE 3

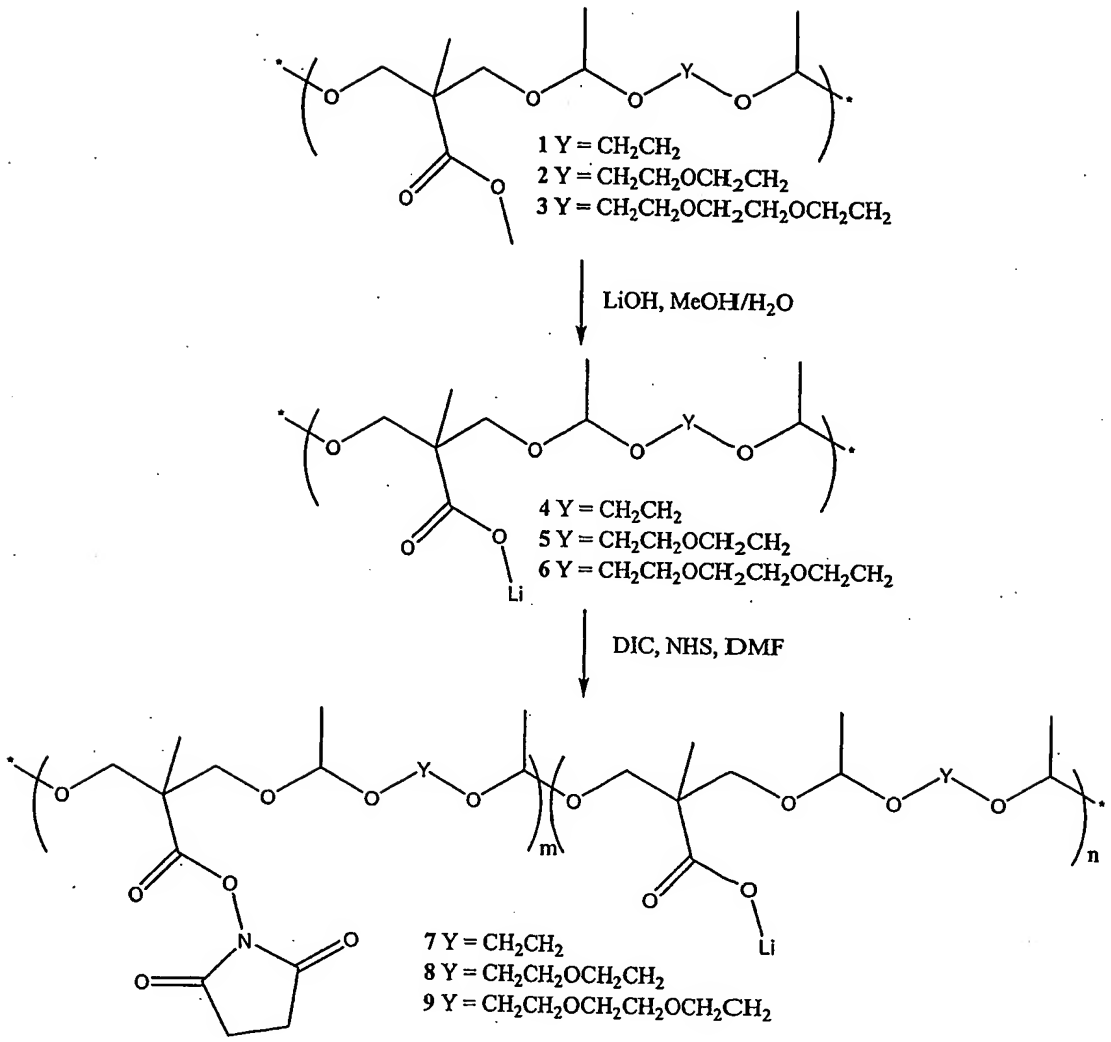

1 Y = CH2CH2
2 Y = CH2CH2OCH2CH2
3 Y = CH2CH2OCH2CH2OCH2CH2
LiOH, MeOH/H2O
4 Y = CH2CH2
5 Y = CH2CH2OCH2CH2
6 Y = CH2CH2OCH2CH2OCH2CH2
DIC, NHS, DMF
7 Y = CH2CH2
8 Y = CH2CH2OCH2CH2
9 Y = CH2CH2OCH2CH2OCH2CH2

FIGURE 4

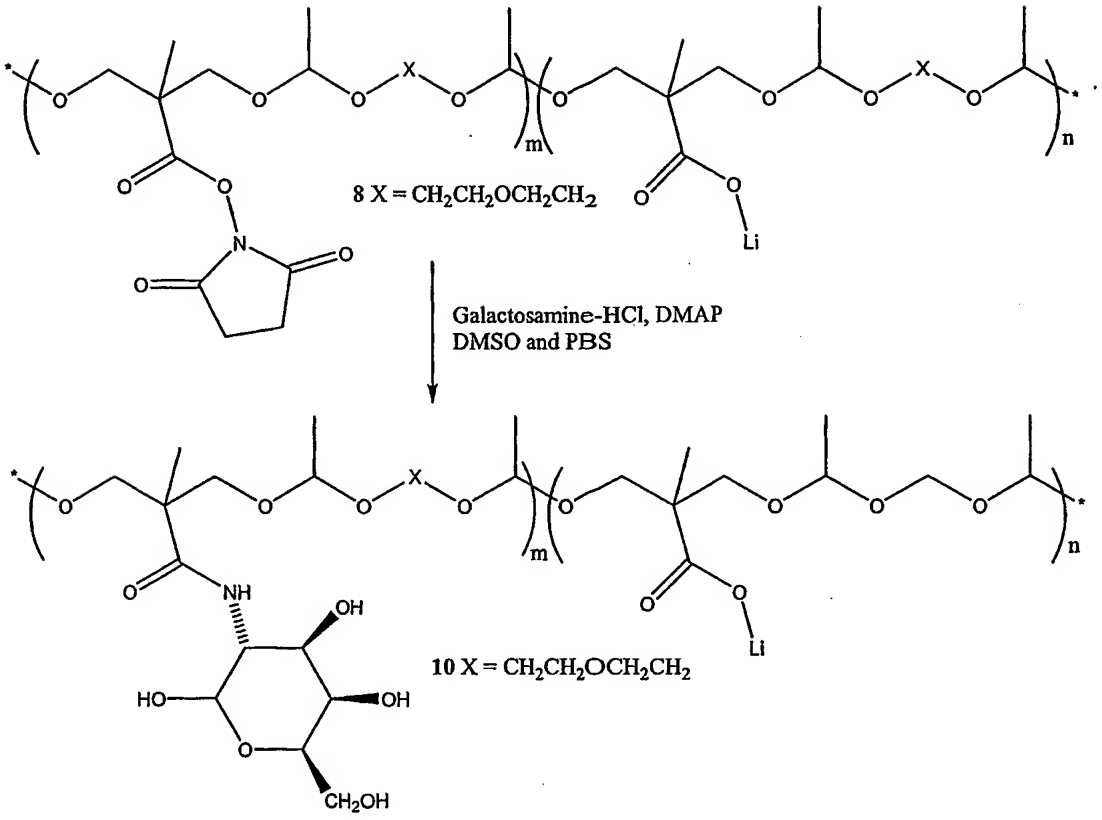

8 X = $CH_2CH_2OCH_2CH_2$
Galactosamine-HCl, DMAP
DMSO and PBS
10 X = $CH_2CH_2OCH_2CH_2$

FIGURE 5

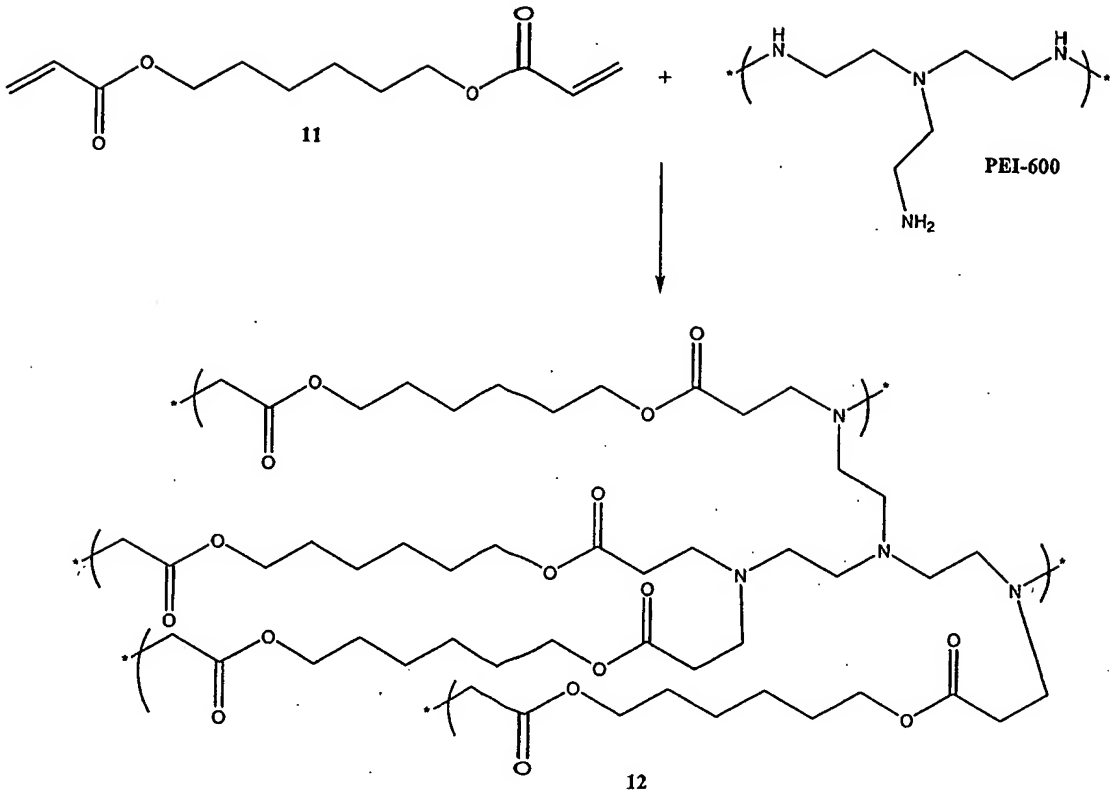


FIGURE 6

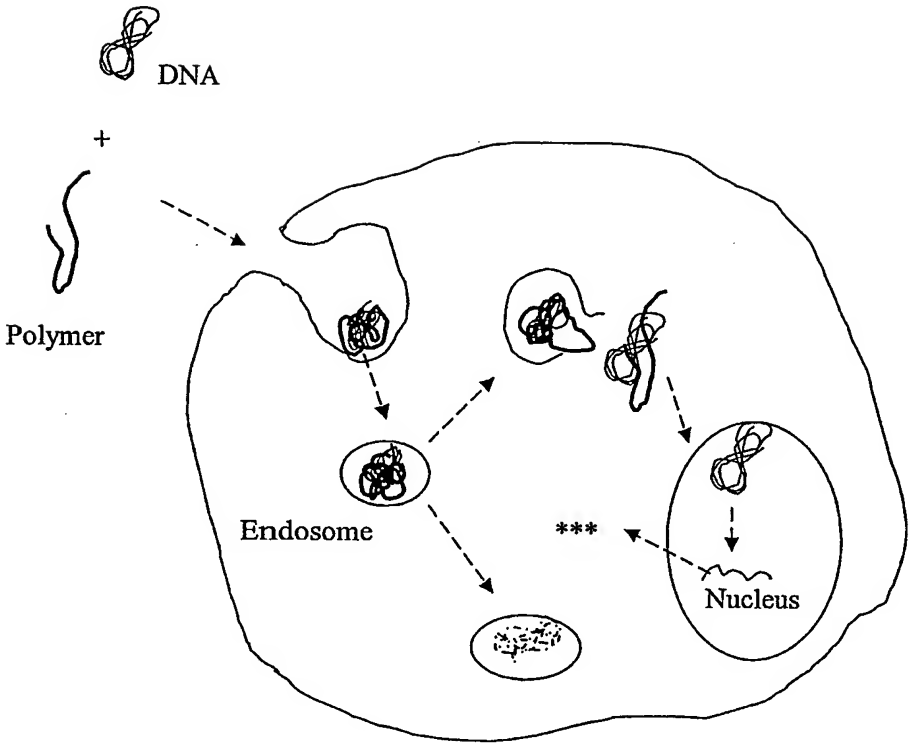


FIGURE 7

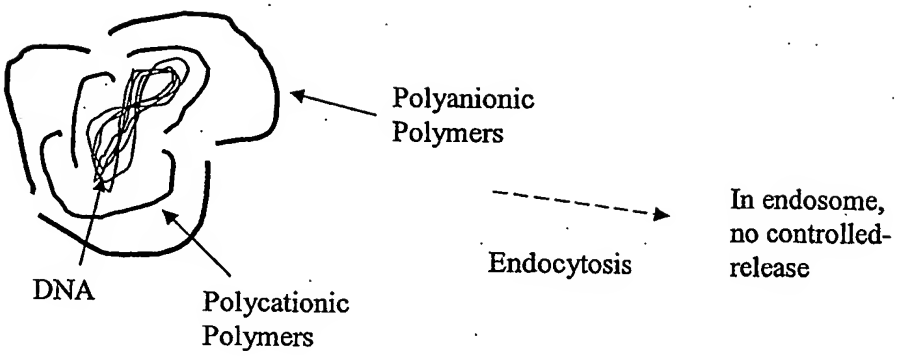

Polyanionic
Polymers
In endosome,
no controlled-
release
Endocytosis
DNA
Polycationic
Polymers

FIGURE 8

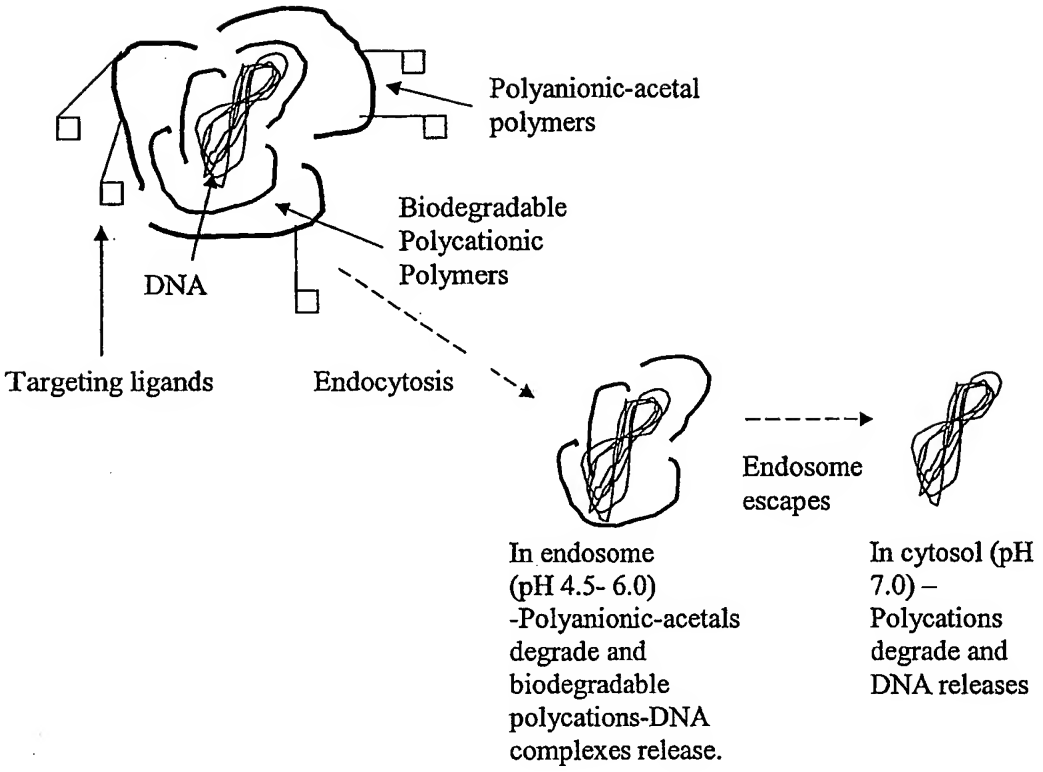
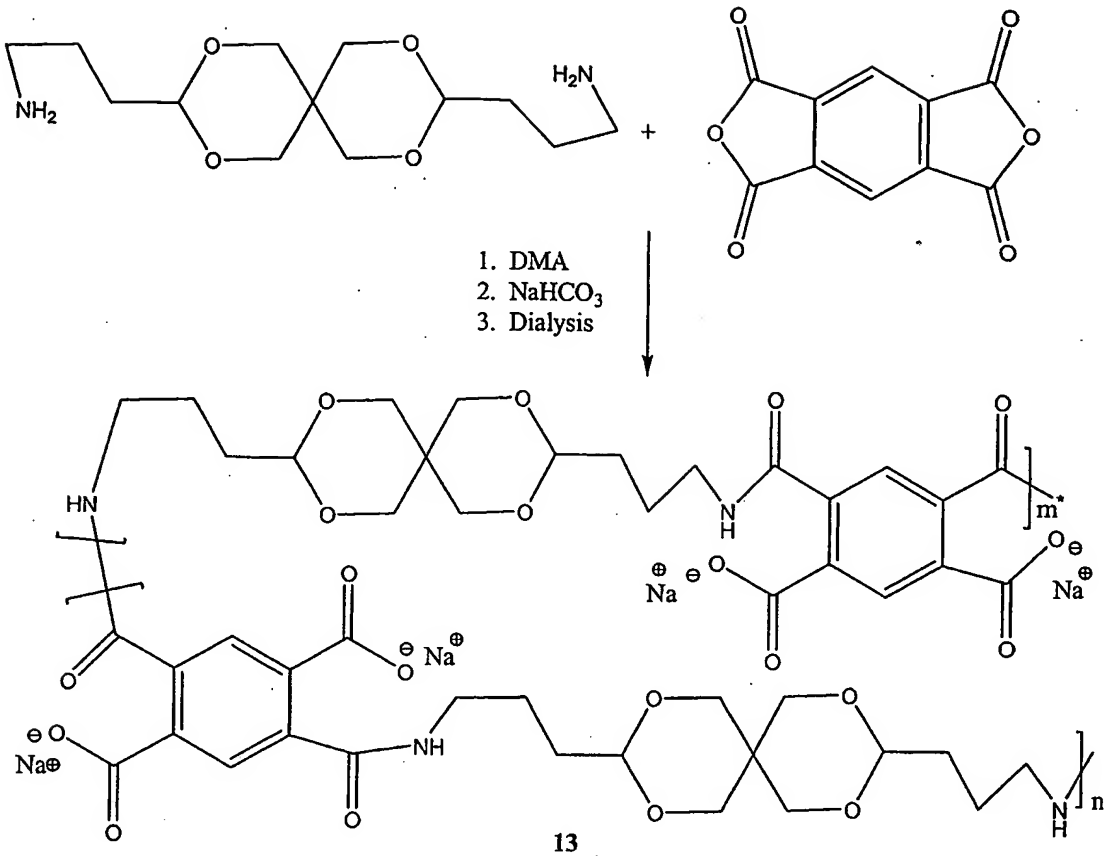

Polyanionic-acetal polymers
Biodegradable Polycationic Polymers
DNA
Targeting ligands
Endocytosis
Endosome escapes
In endosome (pH 4.5- 6.0) -Polyanionic-acetals degrade and biodegradable polycations-DNA complexes release.
In cytosol (pH 7.0) – Polycations degrade and DNA releases

FIGURE 9



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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/030323

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K48/00 C12N15/87 C08L59/00 C08L59/02 C08G4/00
C08G2/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TRUBETSKOY V S ET AL: "Recharging cationic DNA complexes with highly charged polyanions for in vitro and in vivo gene delivery." GENE THERAPY, vol. 10, no. 3, February 2003 (2003-02), pages 261-271, XP002314302 ISSN: 0969-7128 page 261 abstract page 261, right-hand column, paragraph 2	
A	WO 96/32419 A (GEN HOSPITAL CORP) 17 October 1996 (1996-10-17) page 2, line 4 - line 15 page 5, line 8 - line 18 page 6, line 3 - page 9, line 33	

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 January 2005	02/02/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

Int[illegible]al Application No
PCT/US2004/030323

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 2004/076557 A (NITTO DENKO CORP ; JI SHOUPING (US); MATSUMOTO KENJI (US); VAN SANG (U) 10 September 2004 (2004-09-10) claims 1-20	1-22

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

ıational application No.
PCT/US2004/030323

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 14, 15 (both partially), and claims 16-18 (all completely) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ›nal Application No
PCT/US2004/030323

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9632419	A	17-10-1996	US	5811510 A	22-09-1998
			AT	202573 T	15-07-2001
			AU	5531296 A	30-10-1996
			CA	2215997 A1	17-10-1996
			DE	69613569 D1	02-08-2001
			DE	69613569 T2	16-05-2002
			DK	820473 T3	22-10-2001
			EP	0820473 A1	28-01-1998
			GR	3036696 T3	31-12-2001
			JP	11503481 T	26-03-1999
			WO	9632419 A1	17-10-1996
			US	5958398 A	28-09-1999
			US	5863990 A	26-01-1999
WO 2004076557	A	10-09-2004	US	2004166089 A1	26-08-2004
			WO	2004076557 A1	10-09-2004